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Award Number: W81XWH-11-1-0380

TITLE: Validation of Biomarkers for Prostate Cancer Prognosis

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REPORT DATE: December 2014

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| December 2014 | Final | 30 Sep 2011 - 29 Sep 2014 |

**4. TITLE AND SUBTITLE**
Validation of Biomarkers for Prostate Cancer Prognosis

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-11-1-0380

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Stanford University
Stanford, CA, 94305.

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Our objective is to create a multi-institutional tissue microarray resource from radical prostatectomy samples with detailed clinical information and follow-up and rigorous case-cohort design for use as a platform for validating tissue biomarkers of prognosis. In addition, we have proposed testing a series of biomarkers of prognosis and a set of biomarkers that correlate with Gleason Score. We have made significant progress over the past year. Having completed construction of the tissue microarrays and finalized standard procedures for tissue microarray storage, sectioning and shipping, we have now stained scanned, gridded and read TMAs for several biomarkers. We havenow changed to the Leica scanner and PathXL image analysis software suite for some of the biomarkers and have also used the Aperio system for others. Pathologists have read compete sets of TMAs for H & E, High Molecular Weight Keratin, ERG, SPKINK1, Ki67 (MIB1), Survivin and PTEN FISH and we have correlated staining results with clinical outcome. We also have made significant progress in testing TACOMA, an automated TMA scoring algorithm. We have completed staining of the TMAs for . Over the next year we will complete refinements of the infrastructure, complete pathologic review of the p27 and MUC1 biomarkers, and stain and evaluate several additional biomarkers which have received approval. We will complete statistical analysis for all of the completed biomarkers (and others evaluated over the next year) and plan to publish papers for each of the biomarkers over the next year. We wil also carry out outcome analysis for a panel of the biomarkers soon.

**15. SUBJECT TERMS none provided**

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| | | | | | USAMRMC |
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | 19b. TELEPHONE NUMBER *(include area code)* |
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**Validation of Biomarkers for Prostate Cancer Prognosis**

***Final report***

***James D. Brooks***

Synergy Award: W81XWH-11-1-0380

Co-PIs: James D. Brooks & Ziding Feng

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## Introduction

The most significant challenge in managing localized prostate cancer is the decision of whether or not it needs to be treated. Nearly ½ of prostate cancers diagnosed in the U.S. fall into the low or very low risk category and have little likelihood of causing death. However, it is well known that a significant fraction of low risk cases are misclassified and actually have occult high-risk features or are destined to progress to high-risk disease. Therefore a critical need in localized prostate cancer is the development of biomarkers that predict occult or incipient aggressive disease in the low-risk population.

To address this challenge, we formed the multi-institutional Canary Tissue Microarray Project. We have used rigorous clinical trial case/cohort design, taking care to correct for institutional and spectrum biases. Funding from the Department of Defense allowed us to complete construction of the TMAs as well as the necessary infrastructure and begin testing biomarker candidates. With this infrastructure in place, we now have a robust validation platform for testing prostate cancer biomarkers. Based on our success, this resource will be a source for future biomarker validation studies even after the DOD funding has ceased.

The DOD has catalyzed the formation of the infrastructure to support this project and we have now completed or are near completion of several biomarkers. Staining has been completed, slides have now been analyzed and statistical analyses are underway. My collaborator in this Synergy Award, Ziding Feng, has requested and received an extension of his half of the award because of his move from University of Washington to MD Anderson Cancer Center. Actually, this will be very beneficial for this project since the next phase for several of the biomarkers is the statistical analysis of the data. His work over the next year will complete several projects and should lead to several publications. This will serve as critical preliminary data for us to continue this resource and apply for competitive funding.

## Specific Aim 1) To test markers of prognosis on prostate cancer tissue microarrays with associated clinical data.

### 1.A. Develop work-flow for TMA sharing, image scanning, TMA staining data analysis.

As stated last year, the multi-institutional TMAs have been constructed at all sites, with a final TMA cohort of 1326 patients, of which 1232 have clinical data. Details of patient selection, statistical considerations, and TMA construction are summarized in our publication in Advances in Anatomic Pathology published in 2013. In addition to this cohort, a separate TMA has been constructed from 220 patients who underwent radical prostatectomy at a sister site who have very long term follow-up (up to 25 years) and hard endpoints including metastases and prostate cancer specific death. Since many of these patients were diagnosed in the pre-and early PSA eras, they are held separately as a validation cohort.

We have completed the stated aims in the proposal with regard to development of work-flow for array sharing, analysis and archiving. We also learned that some of the aspects of our original design were not feasible or not efficient. A summary of our successes and learning points is below.

1) After TMA manufacture was completed, Standard Operating Procedures (SOPs) for TMA storage, sectioning and transferal have all been working well at each site. Staining for the biomarkers currently under evaluation has been excellent, with the exception of cases from the Eastern Virginia Medical Center. We are evaluating the possible differences in fixation or sample preparation that could account for the staining performance. Regardless, we still have ample power from the remaining cases (approximately 1000) for meaningful validation of biomarkers.

2) Slide shipping works well for sending slides to investigators for staining, as well as to the image scanning centers.

3) We have an established monthly conference call for trouble-shooting, assigning tasks such as slide reading, reviewing proposals for use of the resource, review of data, and work on publications. These phone calls will continue despite the end of funding from the DOD.

4) We have completed H & E staining of the complete set at Stanford University. In addition, we have stained the complete set for high molecular weight keratins (HMWK) to aid the pathologists in interpreting slides. These have been reviewed and scored by a single pathologist (Jesse McKenney at the Cleveland Clinic) and are available to all pathologists for ascertainment whether cancer is present in the core. This saves the pathologists considerable time when reviewing staining for any of the candidate biomarkers since they will not score missing cores or cores in which the cancer has run out as we have sectioned more deeply into the block.

5) Image capture and archiving has been exhaustively evaluated. This aspect of the project has proven very challenging. In the original proposal, we had proposed using the Stanford Tissue Microarray Database (STMAD) for scanned images and archiving. Unfortunately the scanning platform compatible with the STMAD required 24 hours per slide for scanning, which was simply not practical since a single set of TMAs encompasses 34 slides – scanning time for a single marker would take more than 1 month. We therefore switched to the Leica SCN400 Slide Scanner with the SL801 Autoloader based at University of British Columbia. The Leica scanner has the advantage of speed, for it can capture high resolution images of an entire TMA in about 1 hour and is fully automated so a deck of slides can be loaded and scanned.

Since we could not use STMAD, we decided to use the PathXL image analysis software suite (http://www.pathxl.com/index.php/pathxl-research/pathxl-tma). This system appeared to be ideal since it allowed us to set up scoring parameters and image manipulation that STMAD lacked. Unfortunately, the image interface was very slow (in loading images) and did not allow the pathologists to look at multiple images at once.

The latter problem was particularly acute since the pathologists often wanted to look at the H & E image, HMWK and IHC image simultaneously in order to delineate the cancer region in each core and only score that area for IHC staining. Therefore, this system was not acceptable to the pathologists. In the end, the pathologists have resorted to scoring the slides either by directly reading the images scanned from the Leica scanner, or simply reading the glass slides (the actual stained sections) using the microscope and recording their reads into an Excel database. This proved to be the most time-efficient and practical solution for the pathologists. Having solved this issue we have now moved along to testing biomarkers.

6) One major challenge has been the considerable time required of the pathologists to simply read the TMAs. As mentioned above our TMAs have 1326 patients represented, each with 4 cores. In other words: **1326 pts (x 4 cores) = 5304 cores**. This is a considerable number of cores for the pathologists to read. If they also include H & E and HMWK the work becomes overwhelming, i.e.: **1326 pts (x 4 cores)= 5304 cores (x 3 stains) = 15912 stains**. Regardless, the reading of 5304 cores requires a single pathologist on average approximately 70 hours to look at and score all of the cores. This time commitment is significant, especially considering that the pathologists are not being paid from this or any grant to perform the reads. And yet we have completed staining and reads for several biomarkers (see below).

7) We have looked at inter-observer variability in reading IHC stains for ERG. In this experiment, we had 7 pathologists score one TMA (200 cores) for ERG staining, a biomarker with highly robust and reproducible staining. In the first round, pathologists scored the TMAs according to their own systems – without prior discussion of the methods they would use for assessing positive, intermediate and negative cores. The agreement was good, but modest. In a second round, pathologists agreed upon scoring metrics and the concordance increased significantly, with near complete agreement between the pathologists.

8) Data management: The clinical data are complete for the TMAs and have been used by Dr. Feng for analysis of staining results of the TMAs, as detailed in his report. As mentioned above, Dr. Feng has moved from Fred Hutchinson Cancer Research Institute to MD Anderson Cancer Center. As a result, he has applied for and received an extension of his half of the funding. As mentioned above, this is highly advantageous to the success of this project because of delays in the arrival of staining data (due to issues outlined above). Since we have now resolved production issues, Dr. Feng's group is now awash with data and the continued funding will allow him to complete the analyses so we can report our important findings. He will discuss the transfer of the DMCC to MDACC.

9) TACOMA progress will be reviewed by Dr. Feng in his report.

**1.B. Test candidate biomarkers of prognosis for prediction of recurrence after radical prostatectomy**

In our ongoing monthly conference calls, the TMA investigators review progress and review applications for utilizing the TMAP resource. Most applications for use of the TMAs come from within the group, although it is available to the prostate cancer research community broadly and can be accessed by application through the Canary Foundation website (http://www.canaryfoundation.org). We have focused on biomarkers that have well characterized, highly performing reagents (e.g. immunohistochemical grade antibodies) and sufficient preliminary data that they could supply prognostic information independent of grade, stage and PSA. We have now completed staining for many of the biomarkers listed in our proposal and are expanding to novel biomarkers discovered since our application.

Completed biomarkers:
1) ERG: Immunohistochemistry for the ERG protein has been completed, scored and is being analyzed by the DMCC. Preliminary data show that ERG staining does not provide prognostic information either on univariate or multivariate analysis. However, we are in the process of redoing these analyses trying to correct for some biases in the length of follow-up for the cases. A manuscript is quite far along and will be submitted in the next few months.

2) SPINK1: As reported previously, SPINK1 positive tumors constitute a minority of prostate cancer – in the Canary TMA only 6% of cases. In addition, positive staining is confined to the ERG-fusion negative cases, with 2 exceptions in our dataset. However, unlike previous data, SPINK1 high level expression appears to be correlated with favorable outcome in that it is associated with higher recurrence free survival RFS in a preliminary analysis. The final statistical analysis is being completed by Dr. Feng's group. We will be reporting the ERG and SPINK1 results in a single manuscript in the next few months.

3) PTEN FISH: In collaboration with Dr. Jeremy Squire at Queens University, Ontario, Canada, we have used a multiprobe FISH assay to interrogate copy number alterations (allelic loss) at the PTEN locus. In our series, homozygous deletion of PTEN was found in 9% of cases and heterozygous allelic loss was found in an additional 9% of cases. PTEN loss was associated with adverse pathology including extracapsular extension, seminal vesicle invasion and lymph node spread. In addition, allelic loss events of any type were associated with poorer RFS. Finally, tumors with homozygous deletion appear to have more aggressive features than those with hemizygous deletion or no structureal alterations at the PTEN locus. A manuscript has been submitted to "The Prostate".

4) ERG IHC and PTEN IHC: In collaboration with Tamara Lotan at Johns Hopkins, we completed IHC staining for PTEN on our TMAs. There was excellent agreement between the PTEN IHC results and PTEN FISH. IHC has the advantage of working in a larger number of cores than FISH so we were able to carry out a more complete

evaluation of the cohort. Again, PTEN loss was associated with adverse outcome. Moreover, PTEN loss was associated with poor outcome to a much greater degree in the ERG fusion negative cases as opposed to the ERG positive cases. This work will be presented at several up-coming international meetings. A manuscript has been completed and is being revised for submission in the next month.

5) Ki67: Ki67staining has been used as a measure of proliferative index and has been shown to be prognostic in several tumor types including prostate cancer. However, since prostate cancer has a low proliferative index (PI), and there is considerable inter-observer variation of interpretation of Ki67 stains, we decided to use an automated imaging process to score Ki67 staining. We used the Aperio system to quantify stained and unstained nuclei in regions of prostate cancer across 1000+ samples on our TMA. Ki67 PI was significantly associated with adverse pathologic features and RFS in univariate and multivariate analysis. High Ki67 PI was also associated with overall survival and prostate cancer specific survival in this cohort. It appears to be an excellent prognostic biomarker. A manuscript has been drafted and final comments are being assembled. It should be submitted within the next 1 month.

6) AZGP1: AZGP1 has been shown to be prognostic in several datasets and was originally described by the Brooks group in 2004. We have performed both IHC and RNA ISH for AZGP1 and the TMAs have been scored. An initial analysis has been completed by the DMCC and is currently being revised. A preliminary look at the data shows that AZGP1 positive IHC staining is correlated with a lower risk of RFS. When this analysis is completed, we expect a manuscript to be submitted before the end of the calendar year.

7) Ongoing studies: We have completed staining and pathologist reads for CD38, p63, CD10, and Muc1. We have also completed a project in image analysis of H & E slides with Gustavo Ayala at University of Texas. Finally, we have completed an analysis of a radical modification of the Gleason scoring system with Jesse McKenney at the Cleveland clinic. Each of these projects needs to be analyzed by the DMCC now that the data have been acquired. In addition, we have ongoing pathologist reads going for ARG2, p27 (using the Aperio system) SMAD7 and Trichrome stain for stromal desmoplastic reaction. Once these are completed they too will be sent to the DMCC. We also have 4 additional projects approved and are about to cut new sections for these projects. We expect the next 2 years to be highly productive.

**Specific Aim 2) To evaluate candidate markers that correlate with Gleason grade on prostate cancer tissue microarrays with associated clinical data.**

Thus far, we have focused on building the analysis pipeline and in staining high priority biomarkers of prognosis. In all of the biomarkers we have tested thus far, we have interrogated each for its correlation with Gleason score. In general, most have correlated, although not completely. While these do not address the intent of this Aim, we are not disappointed since it does appear that *these biomarkers are supplying prognostic information that is independent of Gleason score.* The intent of Aim 2, on the

other hand, was to investigate biomarkers that correlate with Gleason grade. Several markers are in our queue and are listed in the original proposal. For some, we are still looking for high quality affinity reagents that provide interpretable staining with limited background. Leading candidates are AGR2, a marker expressed at high levels in Gleason pattern 3 cancers and Monoamine oxidase A, expressed at high levels in Gleason pattern 4 disease. As we get through our candidate prognostic markers (listed above and in the queue) we will refocus on biomarkers that predict Gleason grade. This could be useful in characterizing biopsy samples to predict upgrading.

However, this clinical question might become less relevant in the future since several tools have been developed that already predict up-grading. For example the OncotypeDx assay has been calibrated and already validated precisely for this purpose. In addition, multiparametric MRI shows good correlation with grade in that only the high-grade lesions are visible, while the low grade lesions are not. As the clinical practice evolves, we will decide whether we wish to continue to pursue development of IHC biomarkers that predict Gleason score

For all biomarkers, whether for Gleason score or prognosis, the statistical analysis strategy has been outlined in our proposal and will be used as soon as reads are available from the pathologists.

**Key Research Accomplishments**

- Completion of construction of TMAs at all participating sites
- Standardizing and deploying Standard Operating Procedures for TMA storage, sectioning and shipping at each site
- Centralized shipping, collation and distribution of TMAs at Stanford University
- Biomarker review and approval by the investigative team to ensure quality of the reagents and sufficient level of evidence for investigation of a particular biomarker on our valuable resource.
- Inclusion of investigators in the broad prostate cancer research community for testing candidate biomarkers. Groups using the resource include Dr. Jeremy Squire, Dr. Gustavo Ayala, Tamara Lotan and Dr. Lidong Liu.
- Porting final clinical data that will be used for analysis of biomarker performance to the MD Anderson DMCC.
- Deployment of a more efficient image capture system (Leica) so that we can increase the throughput of biomarker testing.
- Use of the Aperio image analysis system with Ki67 (MIB1) with plans to adapt to p27 (KIP1)
- Testing of a new image archiving and displaying software for management and scoring of the immunohistochemical staining by the study pathologists
- Completion of foundational staining for H & E and HMWK. Ina ddition, we have completed the pathologist interpretation of the cores for each of these stains and are incorporating these in the database to be made available for the patholosts to use in interpreting each core on the TMA for new stains.
- Completion of analysis of PTEN FISH and submission of a manuscript
- Completion of analysis of Ki67 PI and imminent submission of a manuscript
- Completion of analysis of ERG IHC and PTEN IHC and presentation at international meetings and imminent submission of a manuscript.
- Ongoing analysis of ERG and SPINK with a manuscript near completion.
- Ongoing analysis of AZGP1 with a manuscript expected soon.
- Ongoing analysis of image analysis with Gustavo Ayala.
- Ongoing analysis of a modified Gleason grading system with Jesse McKenney, as well as confirmation in an additional validation set.
- Ongoing analysis of Muc1, p63, CD10 and CD38. We expect all of these, regardless of outcome (prognostic or not) will be submitted as separate publications.
- Significant preliminary data from this collaboration that will position us well for the next phase of funding.

## Reportable Outcomes

1) Publications referencing this grant:

James D. Brooks: Translational genomics: The challenge of developing cancer diagnostic biomarkers. *Genome Research* **22:** 183-187, 2012.

Sarah Hawley, Ladan Fazli, Jesse K. McKenney, Jeff Simko, Dean Troyer, Marlo Nicolas, Lisa F. Newcomb, Janet E. Cowan, Luis Crouch, Michelle Ferrari, Javier Hernandez, Antonio Hurtado-Coll, Kyle Kuchinsky, Janet Liew, Rosario Mendez-Meza, Elizabeth Smith, Imelda Tenggarra, Xiaotun Zhang, Peter R. Carroll, June M. Chan, Martin Gleave, Raymond Lance, Daniel W. Lin, Peter S. Nelson, Ian M. Thompson, Ziding Feng, Lawrence D. True and James D. Brooks: Design and construction of a resource for the validation of candidate prognostic biomarkers: the Canary Prostate Cancer Tissue Microarray as a model. *Advances in Anatomic Pathology* **20:** 39-44, 2013.

James D. Brooks: Managing localized prostate cancer in the era of prostate specific antigen testing. *Cancer* **119:** 3906-3909, 2013.

Zuxiong Chen, Zulfiqar G. Gulzar, Catherine A. St. Hill, Bruce Walcheck, James D. Brooks: Increased expression of *GCNT1* is associated with altered *O*-glycosylation of PSA, PAP and MUC1 in human prostate cancers. *Prostate* **74:** 1059-1067, 2014.

## Conclusion

We have undertaken a challenging task of creating a multi-institutional TMA resource with rigorous case/cohort design. To our knowledge, such a resource has not been previously created and offers the advantage of reducing institutional biases as well as spectrum biases. In the uniform design and through image acquisition and archiving technologies, we have created a resource that can be easily used by the greater prostate cancer research community. In many ways, this resource represents a gold standard by for evaluation of prognostic biomarkers. We have completed all phases of pipeline construction and have now validated its utility in testing several biomarkers. We have also shown that several types of analytes work well on this resource including immunohistochemistry, In situ hybridization to analyze DNA copy-number alterations, and RNA in situ hybridization. We also have tested several biomarkers and confirmed that they are prognostic. We will complete analysis of the biomarkers in the context of the clinical data over the next year and plan several publications. In addition, we will continue to carry out analysis of new biomarkers and solicit applications for biomarkers inside and outside our research group. This research directly addresses the PCRP overarching challenge to *distinguish lethal from indolent disease*.